UTS UNIVERSITY OF TECHNOLOGY SYDNEY

Novel customisable phases for micro solid-phase extraction and automated biological sample preparation

by Karen Duong

Thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

under the supervision of Distinguished Professor Philip Doble, Dr David Bishop and Dr Raquel Gonzalez de Vega.

University of Technology Sydney
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Certificate of authorship and originality

I, Karen Duong declare that this thesis, is submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the School of Mathematical and Physical Sciences at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise referenced or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

This document has not been submitted for qualifications at any other academic institution.

This research is supported by the Australian Government Research Training Program.

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Abstract

Protein biomarkers play an important role in clinical settings as they serve as measurable indicators for normal or abnormal biological processes. They aid in accelerated disease identification, diagnosis, prognosis, and response to treatments. Recently, mass spectrometry (MS) assays have been introduced as an alternate to the conventionally used immunoassays. MS provides accurate and precise quantification due to its high sensitivity and specificity. However, major challenges lie with sample preparation involving lengthy workflows, limited automation, and challenges related to highly complex biological samples where several techniques are applied.

This thesis aims to improve sample preparation techniques to provide an accurate, rapid, and automated method for protein biomarker quantification using micro-solid-phase extraction (μSPE) technology through the development of a micro-immobilised-enzyme reactor (IMER) and a μSPE immunoaffinity cartridge to alleviate sample preparation bottlenecks caused by conventional ~18-hour digestions.

A novel and customisable material was prepared for bio-ligand immobilisation. A hybrid inorganic-organic material using silica modified with carboxymethylated dextran (CMD) was prepared. This material was packed into μSPE cartridges and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS) coupling was exploited to immobilise enzymes and antibodies in-situ using a programmable syringe driver allowing for the development of an automated workflow.

Reproducible trypsin digestion was observed using high performance liquid chromatography (HPLC) through the cleavage of N-α-Benzoyl-L-arginine ethyl ester (BAEE). Three model proteins (bovine serum albumin, cytochrome c and thyroglobulin) and a human serum sample were analysed, and compared to conventional in-solution digestion by employing liquid chromatography orbitrap mass spectrometry (LC-OT-MS) and liquid chromatography quadrupole time of flight mass spectrometry (LC-QToF-MS). The IMER facilitated protein digestion within 10 minutes at room temperature and overall, observed lower sequence coverages and number of identified proteins compared to the conventional in solution digestion method at 37°C.

In the same manner as trypsin, anti-BSA was immobilised and BSA isolation was confirmed using size exclusion chromatography hyphenated to triple quadrupole inductively coupled plasma mass spectrometry (SEC-ICP-MS/MS). Whilst immobilisation of anti-BSA was achieved, challenges with low level protein detection were observed.

The immunoaffinity and trypsin μSPE cartridge were combined into an automated workflow for protein pre-concentration and digestion. Using BSA as the model protein, BSA standards and BSA spiked into human serum samples were subjected to the workflow. BSA extraction and digestion was achieved, however, the complex human serum matrix negatively impacted the BSA isolation compared to neat standards. Further investigation and optimisation of the workflow must be performed.

List of Publications and Presentations

Journal Publications

Immunoaffinity extraction followed by enzymatic digestion for the isolation and identification of proteins employing automated µSPE reactors and mass spectrometry (Submitted to Analytical and Bioanalytical Chemistry).

Application Notes

Karen Duong, Simin Maleknia, Andrew Minett, David Bishop, Philip Doble, "µSPEed-Cxyl microreactor cartridges: Trypsin cartridges for digests of bovine serum albumin (BSA)", ePrep Application Note, 2019.

Karen Duong, Philip Doble, "µSPEed recovery curve with 4 nitrotoluene", ePrep Application Note, 2015.

Conference Poster Presentations

Karen Duong, Raquel Gonzalez de Vega, Andrew Minett, David Bishop, Philip Doble, *"Immunoaffinity and enzymatic reactor micro-solid-phase extraction cartridges for rapid protein isolation and digest"*, American Society for Mass Spectrometry Conference, 2019, Atlanta, Georgia, USA.

Karen Duong, Simin Maleknia, Andrew Minett, David Bishop, Philip Doble, *"Immobilised enzymes on micro-solid-phase extraction cartridges for automated protein digestion"*, American Society for Mass Spectrometry Conference, 2018, San Diego, California, USA.

Karen Duong, Simin Maleknia, Andrew Minett, David Bishop, Philip Doble, *"Customisable micro-solid-phase extraction for selective binding in biological sample preparation"*, The Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, 2018, Orlando, Florida, USA.

Karen Duong, Simin Maleknia, Andrew Minett, David Bishop, Philip Doble, *"Immobilised enzymes on customisable micro-solid-phase extraction cartridges for proteolytic digests in minutes"*, Lorne Proteomics Symposium, 2018, Lorne, Melbourne, Australia.

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List of Abbreviations and Acronyms

Ab-Ag: antibody-antigen complex

ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

ACN: acetonitrile

AGC: automated gain control

APS: aminopropyl silica

ATR: attenuated total reflectance

BA: N_{α}-Benzoyl-L-arginine

BAEE: N_{α}-benzoyl-L-arginine ethyl ester

BCA: bicinchoninic acid

BEC: background equivalent concentration

BIN: barrel insert and needle

BSA: bovine serum albumin

CID: collision induced dissociation

CMD: carboxymethylated dextran

Cyt c: cytochrome c

CV: coefficient of variation

Da: dalton

DMSPE: dispersive micro-solid-phase extraction

DNA: deoxyribonucleic acid

DSS: disuccinimidyl suberate

DTT: dithiothreitol

EDC: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide

ELISA: enzyme-linked immunosorbent assays

ESI: electrospray ionisation

F_{ab}: fragment antigen binding

F_c: fragment crystallisable

FDR: false discovery rate

FI: flow injection

FTIR: Fourier-transform infrared spectroscopy

GC: gas chromatography

HAPs: high abundant proteins

HPLC: high performance liquid chromatography

HRP: horseradish peroxidase

IA: iodoacetamide

IASPE: immunoaffinity-based solid-phase extraction

ICP-MS: inductively coupled plasma mass spectrometry

ID: inside diameter

Ig: immunoglobulin

IMAC: immobilised metal affinity chromatograph

IMER: immobilised enzyme reactor

ITSP: instrument top sample preparation

KHP: potassium hydrogen phthalate

LAPs: low abundant proteins

LC: liquid chromatography

LAC: lectin affinity chromatography

LLE: liquid-liquid extraction

mAb: monoclonal antibody

MALDI: matrix assisted laser desorption/ionisation

MEPS: microextraction by packed sorbents

MES: 2-(*N*-morpholino)ethanesulfonic acid

MIPs: molecularly imprinted polymers

MISPE: molecularly imprinted solid-phase extraction

MOAC: metal oxide affinity chromatography

MRM: multiple reaction monitoring

MS: mass spectrometry

MS/MS: tandem mass spectrometry

MSPE: magnetic solid-phase extraction

MW: molecular weight

m/z: mass-to-charge ratio

NHS: *N*-hydroxysuccinimide

OAI: oriented antibody immobilisation

OD: outside diameter

OT: orbitrap

pAb: polyclonal antibody

PBS: phosphate buffer saline

PEG: poly(ethylene glycol)

pI: isoelectric point

PIT: pre-immobilised trypsin

PPT: protein precipitation

PRM: parallel reaction monitoring

Q1, Q2, Q3: quadrupole 1, 2 and 3

Q-OT: quadrupole-orbitrap

QQQ: triple quadrupole

RAI: random antibody immobilisation

RNA: ribonucleic acid

SBSE: stir-bar sorptive extraction

SEC: size exclusion chromatography

SPE: solid-phase extraction

SPME: solid-phase microextraction

SPR: surface plasmon resonance

SRM: selected reaction monitoring

TCEP: tris(2-carboxyethyl)phosphine hydrochloride

TPCK: L-1-tosylamide-2-phenylethyl chloromethyl ketone

TIC: total ion chromatogram

ToF: time-of-flight

UV: ultraviolet

UV-Vis: ultraviolet-visible

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